3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one suppresses hydrogen peroxide-induced cytotoxicity on PC12 cells via activation of MAPK

Nishina, A ; Kimura, H ; Kozawa, K ; Sommen, G ; Favero, F ; Heimgartner, H ; Koketsu, M ; Furukawa, S

Abstract: We newly synthesized organic selenium compounds (5-membered ring compounds) including 2-selenoxo-1,3-thiazolidin-4-ones (compounds A) and 3-alkoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylates (compounds B). To address whether these compounds show antioxidative effects, we also examined their superoxide radical (O2 −)-scavenging effects. Moreover, we examined the effects of compound Aa on the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinases (MAPK/ERK1/2) and suppression of hydrogen peroxide-induced cytotoxicity in rat pheochromocytoma cells (PC12 cells). We evaluated the O2 −-scavenging activities of the compounds by a chemiluminescence method, and activation of ERK1/2 in PC12 cells was evaluated by Western blot analysis. At 166 mol/L, the O2 −-scavenging activities were markedly different among compounds A and B. 3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Aa) exhibited the strongest superoxide anion-scavenging activity among compounds A and B. The concentration necessary for 50% inhibition of the activity (IC50) of compound Aa was 25.9 mol/L. Compound Aa activated ERK1/2 of the PC12 cell, as did ebselen, and suppressed hydrogen peroxide-induced cytotoxicity more potently than ebselen. In addition, the toxicity of compound Aa was less than that of ebselen. From these results, it is assumed that compound Aa is a candidate drug to prevent oxidative stress-induced cell death.

DOI: https://doi.org/10.1177/1091581811419267

Int. J. Toxcol.
Regular Article

3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one suppresses hydrogen peroxide-induced cytotoxicity on PC12 cells via activation of MAPK

Atsuyoshi Nishina[a,*], Hirokazu Kimura[b], Kunihisa Kozawa[b], Geoffroy Sommen[c], Francesco Favero[d], Heinz Heimgartner[e], Mamoru Koketsu[f], Shoei Furukawa[g]

[a]*Yonezawa Women's Junior College, 6-15-1 Tohrimachi, Yonezawa, Yamagata 992-0025, Japan;* [b]*Gunma Prefectural Institute of Public Health and Environmental Sciences, 378 Kamioki, Maebashi, Gunma 371-0052, Japan;* [c]*Lonza Braine SA, Chaussée de Tubize 297, B-1420 Braine l'Alleud, Belgium;* [d]*Laboratory of Cancer Pharmacogenomics, "Edo Tempia" Foundation, 13900 Biella, Italy;* [e]*University of Zürich, Institut of Organic Chemistry, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland;* [f]*Division of Instrumental Analysis, Life Science Research Center, Gifu University, Gifu 501-1193, Japan; and* [g]*Laboratory of Molecular Biology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan.*

* Corresponding author: Atsuyoshi Nishina, Yonezawa Women's Junior College, 6-15-1 Tohrimachi, Yonezawa, Yamagata 992-0025, Japan;
Tel: +81-238-22-7330; Fax: +81-238-22-7333
E-mail: nishina@yone.ac.jp

We newly synthesized organic selenium compounds (five-membered ring compounds) including 2-selenoxo-1,3-thiazolidin-4-ones (compounds A) and 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylates (compounds B). To address whether these compounds show antioxidative effects, we also examined their superoxide radical (O_2^-) scavenging effects. Moreover, we examined the effects of compound Aa on the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinases (MAPK/ERK1/2), and suppression of hydrogen peroxide-induced cytotoxicity in rat pheochromocytoma cells (PC12 cells). We evaluated the O_2^- scavenging activities of the compounds by a chemiluminescence method, and activation of ERK1/2 in PC12 cells was evaluated by Western blot analysis. At 166 μM, the O_2^- scavenging activities were markedly different among compounds A and compounds B. 3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Aa) exhibited the strongest superoxide anion-scavenging activity among compounds A and B. The concentration necessary for 50% inhibition of the activity (IC_{50}) of compound Aa was 25.9 μM. Compound Aa activated ERK1/2 of the PC12 cell, as did ebselen, and suppressed hydrogen peroxide-induced cytotoxicity more potently than ebselen. In addition, the toxicity of compound Aa was less than that of ebselen. From these results, it is assumed that compound Aa is a candidate drug to prevent oxidative stress-induced cell death.

Key words: 3-(2,6-dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one; superoxide anion-scavenging activities; PC12 cells; MAPK; phosphorylation

Reactive oxygen species [1] including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) are generated in aerobic cells. [2] Reactive oxygen species may react with various pivotal biomolecules such as DNA and proteins, and the excessive reactive oxygen species generation in the cells may lead to denaturation of the molecules. [3] In addition, endogenous reactive oxygen species generators such as granulocytes, monocytes, macrophages, eosinophils and basophils produce reactive oxygen species and damage the tissue/organ due to abnormal inflammation.[4-6] To avoid toxicity of reactive oxygen species under the normal physiological conditions, the reactive oxygen species production may be regulated.[7] As another effect of reactive oxygen species, recent reports suggest that the low concentration of reactive oxygen species in the cell may regulate signaling pathways.[8]

Superoxide dismutases (SODs), catalase, glutathione peroxidases (GPXs) and some vitamins are representative antioxidants that protect against reactive oxygen species toxicity in cells.[4,5] GPXs are believed to be an important antioxidant enzyme and effectively reduces the toxicity of H_2O_2 *in vitro* and *in vivo*.[5,9] Furthermore, the active domain of GPX essentially contains selenium atoms.[9] Various studies have reported that selenoproteins, including GPX, reduce oxidative stress in cells.[10,11] Thus, various organic selenium compounds may be candidates as reactive oxygen species scavengers.

Ebselen is a five-membered ring selenium-containing heterocyclic compound showing GPX-like activity,[12] and is a synthetic antioxidant with reactive oxygen species scavenger effects.[13] The antioxidant effects of ebselen are due to its selective blockade of leukocyte infiltration and activation, leading to elimination of H_2O_2.[13] This compound is a multifunctional antioxidant and a potential chemopreventive agent in inflammation-associated carcinogenesis.[14] As such, various types of organic selenium compounds may be applicable to the reduction of oxidative stress[15-17] and may be used as anti-oxidative/anti-inflammatory substances for therapeutic drugs against inflammatory diseases (i.e., atopic dermatitis) and stroke.[13] Morey et al. demonstrated that some selenoproteins, such as selenoprotein P, regulate the redox potential in cells, resulting in modulation of various phosphorylation pathways including Ras/mitogen-activated protein kinase (MAPK) signaling.[18] On the other hand, we demonstrated that ebselen also influenced the redox potential in rat pheochromocytoma

cells (PC12) cells and induced activation of MAPK/extracellular signal-regulated protein kinases (ERK1/2) (MAPK/ERK1/2), and neural differentiation via regulation of kinases or phosphatases involved in intracellular signaling.[19] Thus, such selenium compounds are thought to play roles not only as reactive oxygen species scavengers, but also as modulators of intracellular signaling.

Against this research background, we sought to synthesize various organic selenium compounds, such as selenoamides,[17] thio- and selenoureas,[16] and selenocarbamates,[15] in order to evaluate their biological potential. It is already known that most of these compounds are also highly efficient *in vitro* O_2^- scavengers.[15,16] Selenoureas in particular showed pronounced anti-inflammatory effects with low toxicity in various human cell lines.[20] In this study, we newly synthesized five-membered ring compounds such as 2-selenoxo-1,3-thiazolidin-4-one (compound A and its analogues: compounds A**a-d**) and 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylate (compound B and its analogues: compounds B**e-j**), and their superoxide radical (O_2^-) scavenging effects as antioxidants were evaluated by a highly sensitive and quantitative real-time kinetic chemiluminescence method. Moreover, to evaluate the biological effects such as cytotoxicity, cell death inhibition, and cell death/cell proliferation-related signaling pathways of 3-(2,6-dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound A**a**) and ebselen, we examined whether they could activate MAPK/ERK1/2 and suppress H_2O_2-induced cytotoxicity in PC12 cells.

MATERIALS AND METHODS

Materials. Compounds A**a-d** and B**e-j** were prepared according to previously reported procedures .[21,22] A *Cypridina* luciferin analogue, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride (MCLA), obtained from Tokyo Kasei (Tokyo, Japan), was used as a chemiluminescent probe for superoxide radicals. 2-methyl-6-(4-methoxyphenyl)-3,7- dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride was dissolved in twice-distilled water and stored at –80 ºC prior to use. The concentration of 2-methyl-6-(4-methoxyphenyl)-3,7- dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride solution was determined by absorbance at 430 nm using an

absorbance coefficient value of ε= 9,600 $M^{-1}cm^{-1}$, as described.[23] SOD (lyophilized powder, 3400 units/mg protein) and xanthine oxidase (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hypoxanthine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification. All other chemicals and solvents were of analytical grade and used without further purification.

Synthetic methods for the preparation of compounds Aa-d. A round bottom flask equipped with a condenser and a magnetic stirrer was charged with an aryl isoselenocyanate (1.0 mmol) in 20 mL of a mixture of ethyl alcohol and water (2:1). An equimolar amount of the respective mercapto carboxylic acid was then added dropwise. The mixture was stirred at room temperature for several hours before being poured into 50 mL of cold water. After 2 hours of stirring, the precipitate was filtered and purified, if necessary, by recrystallization from ethyl alcohol.

3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Aa). Yield 56%. Orange powder. Mp: 123–125°C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.10 (*s*, 2 Me), 3.95 (*s*, CH_2), 7.18–7.33 (*m*, 3 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 17.5, 37.3, 127.5, 127.9, 130.0, 136.1, 173.2, 200.2. CI-MS: 303 (60, $[M+NH_4]^+$), 286 (100, $[M+H]^+$). Theoretically-calculated values were for $C_{11}H_{11}NOSSe$: C, 46.48; H, 3.90; N, 4.93; S, 11.28. Found: C, 46.33; H, 4.12; N, 5.00; S, 11.32.

3-(4-Methylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ab). Yield 91%. Mp: 201–203°C (ethyl alcohol). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.42 (*s*, Me), 3.90 (*s*, CH_2), 7.10, 7.35 (*AA'BB'*, *J* = 8.2, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 21.3, 37.4, 128.0, 130.3, 133.2, 140.0, 173.2, 203.3. CI-MS: 289 (31, $[M+NH_4]^+$), 272 (100, $[M+H]^+$). Theoretically-calculated values were for $C_{10}H_9NOSSe$: C, 44.45; H, 3.36; N, 5.18; S, 11.87. Found: C, 44.30; H, 3.21; N, 5.03; S, 11.78.

3-(4-Methoxyphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ac). Yield 93%. Mp: 182–184°C (ethyl alcohol).^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.85 (*s*, MeO), 3.89 (*s*,

CH_2), 7.03, 7.14 (*AA'BB'*, *J* = 8.2, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 37.3, 55.4, 114.9, 128.2 (1 arom. C), 129.4, 160.3, 173.3, 203.6. CI-MS: 305 (32, $[M+NH_4]^+$), 288 (100, $[M+H]^+$). Theoretically-calculated values were for $C_{10}H_9NO_2SSe$: C, 41.96; H, 3.17; N, 4.89; S, 11.20. Found: C, 42.23; H, 3.33; N, 4.88; S, 11.12.

3-(4-Bromophenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ad). Yield 89%. Mp: 111–113°C (ethyl alcohol). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.92 (*s*, CH_2), 7.11, 7.67 (*AA'BB'*, *J* = 8.1, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 37.4, 123.3, 130.0, 132.9, 138.5, 171.8, 203.8. CI-MS: 353 (35,$[M+NH_4]^+$), 336 (100, $[M+H]^+$). Theoretically-calculated values were for $C_9H_6NOSSeBr$: C, 32.26; H, 1.80; N, 4.18; S,9.57. Found: C, 32.41; H, 2.02; N, 3.98; S, 10.00.

Synthetic methods for the preparation of compounds Be-j. A 25 mL round-bottom flask equipped with magnetic stirrer and condenser was charged with a mixture of diethyl or bis(tert-butyl) azodicarboxylate (0.92 mL, 2.0 mmol) and Ph_3P (524 mg, 2.0 mmol) in dichloromethane (20 mL). The mixture was stirred under N_2 atmosphere at 0°C (ice bath) for 30 minutes. The appropriate amount of isoselenocyanate (2.0 mmol) was added in one portion. The mixture was stirred for 15 hours at room temperature, and then evaporated to dryness under reduced pressure. The crude product was purified by column chromatography on SiO_2 using hexane/ethyl acetate (100/0 to 50/50) as the eluent, and recrystallized in ethyl acetate.

Ethyl 3-ethoxy-4,5-dihydro-4-phenyl-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Be). Yield: 552 mg (81%). Yellowish crystals. Mp: 142-144°C (ethyl acetate). IR (cm^{-1}): 3422w (br), 2981w, 2931w, 1773s, 1619s, 1595w, 1501w, 1451m, 1386m, 1368m, 1328s, 1308s, 1220s, 1175w, 1155w, 1109w, 1088w, 1066w, 1028m, 1005m, 979w, 902w, 861w, 847w, 776w, 710w, 689w. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.36 (t, J = 7.1, CH_3), 1.49 (t, J = 7.1, CH_3), 4.49-4.62 (m, 2 CH_2), 7.35 (d, J = 8.1, 2 arom. H), 7.52-7.59 (m, 3 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.1, 65.0, 68.5, 128.1, 129.4, 130.0, 132.6, 148.2, 156.3, 167.8. ESI-MS: 360 (14), 361 (13), 362 (52), 363 (3), 364 (100, $[M+Na]^+$), 365 (11), 366 (15). Theoretically-calculated values

were for $C_{13}H_{15}N_3O_3Se$: C, 45.89; H, 4.44; N, 11.86. Found: C, 45.58; H, 4.58; N, 12.64.

Ethyl 3-ethoxy-4,5-dihydro-4-(4-methylphenyl)-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bf). Yield: 554 mg (78%). Yellowish crystals. Mp 120-122°C (ethyl acetate). IR (cm^{-1}): 3442w (br), 2979w, 2956w, 1768s, 1619s, 1514m, 1477w, 1454m, 1389m, 1368m, 1327s, 1307s, 1294s, 1220s, 1173w, 1152w, 1109w, 1099w, 1064w, 1029m, 981w, 905w, 850w, 817w, 753w, 712w, 622w. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.37 (t, J = 7.1, CH_3), 1.49 (t, J = 7.1, CH_3), 2.43 (s, CH_3), 4.48-4.60 (m, 2 CH_2), 7.22, 7.34 (AA'BB', J = 8, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.3, 21.3, 65.0, 68.4, 127.7, 130.0, 127.3, 140.2, 149.0, 156.0, 167.1. ESI-MS: 374 (12), 375 (14), 376 (48), 377 (4), 378 (100, $[M+Na]^+$), 379 (11), 380 (13), 733 (3). Theoretically-calculated values were for $C_{14}H_{17}N_3O_3Se$: C, 47.46; H, 4.84; N, 11.86. Found: C, 47.25; H, 4.85; N, 11.85.

Ethyl 3-ethoxy-4-(4-fluorophenyl)-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bg). Yield: 603 mg (84%). Yellowish crystals. Mp 151-153°C (ethyl acetate). IR (cm^{-1}): 3442w (br), 3085w, 2982w, 1769s, 1621s, 1512s, 1475w, 1454m, 1386m, 1367m, 1311s, 1290s, 1216s, 1171w, 1150w, 1112w, 1088w, 1063m, 1024m, 1004w, 977m, 905w, 853m, 821w, 759w, 725w, 711w, 635w, 622m. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.35 (t, J = 7.1, CH_3), 1.49 (t, J = 7.1, CH_3), 4.41-4.51 (m, 2 CH_2), 6.95, 7.19 (AA'BB', J = 8, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.3, 65.1, 68.6, 116.4, 130.1, 128.4, 148.9, 155.8, 162.3, 167.1. ESI-MS: 378 (15), 379 (12), 380 (55), 381 (4), 382 (100, $[M+Na]^+$), 383 (15), 384 (17). Theoretically-calculated values were for $C_{13}H_{14}N_3O_3SeF$: C, 43.59; H, 3.94; N, 11.73. Found: C, 43.37; H, 4.00; N, 11.88.

Ethyl 4-(4-chlorophenyl)-3-ethoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bh). Yield: 726 mg (97%). Yellowish crystals. Mp 142-144°C (ethyl

acetate). IR (cm^{-1}): 3442w (br), 2982w, 1765s, 1625s, 1495m, 1469w, 1442w, 1373m, 1310s, 1289s, 1218s, 1173w, 1148w, 1089m, 1025m, 977m, 907w, 836w, 753w, 703w. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.31 (t, J = 7.1 Hz, CH_3), 1.41 (t, J = 7.1, CH_3), 4.39-4.51 (m, 2 CH_2), 7.24, 7.43 (AA'BB', J = 8, 2H, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 14.1, 14.3, 65.1, 68.7, 124.6, 129.5, 129.7, 131.0, 148.9, 155.6, 166.9. ESI-MS: 394 (13), 395 (14), 396 (51), 397 (4), 398 (100, $[M+Na]^+$), 399 (18), 400 (49). Theoretically-calculated values were for $C_{13}H_{14}N_3O_3SeCl$: C, 41.67; H, 3.77; N, 11.21. Found: C, 41.63; H, 3.84; N, 11.34.

Ethyl 4-(4-bromophenyl)-3-ethoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bi). Yield: 686 mg (82%). Yellowish crystals. Mp 147-149°C (ethyl acetate). IR (cm^{-1}): 3444w (br), 2982w, 2935w, 1755s, 1718m, 1638s, 1591w, 1493m, 1451m, 1392m, 1366m, 1327s, 1303s, 1286s, 1215w, 1170w, 1154w, 1104w, 1068w, 1030m, 1011m, 988w, 909w, 850w, 754w, 711w. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.31 (t, J = 7.1, CH_3), 1.41 (t, J = 7.1, CH_3), 4.41-4.51 (m, 2 CH_2), 7.20, 7.58 (AA'BB', J = 8, 4 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 14.1, 14.3, 65.1, 68.7, 124.2, 129.8, 132.7, 131.5, 148.9, 155.6, 166.8. ESI-MS: 438 (15), 439 (12), 440 (55), 441 (4), 442 (100, $[M+Na]^+$), 443 (14), 444 (12). Theoretically-calculated values were for $C_{13}H_{14}N_3O_3SeBr$: C, 37.25; H, 3.37; N, 10.03. Found: C, 37.12; H, 3.23; N, 9.99.

tert-Butyl-3-(tert-butoxy)-4-(4-bromophenyl)-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bj). Yield: 855 mg (90%). Yellowish crystals. Mp 118-120°C (ethyl acetate). IR (cm^{-1}): 3443w (br), 2985w, 2931w, 1764s, 1701w, 1612s, 1488m, 1453w, 1408w, 1372m, 1337m, 1320s, 1297m, 1278s, 1223m, 1143s, 1065w, 1000m, 895w, 848m, 836m, 804w, 761w, 655w.^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.53 (s, 3 CH_3), 1.67 (s, 3 CH_3), 7.21, 7.62 (AA'BB', J = 8, 4 arom. H). ^{13}C-NMR: δ 27.6, 27.8, 86.6, 88.1, 123.8, 129.8, 132.5, 132.1, 147.1, 153.5, 165.1. ESI-MS: 494 (17), 495 (15), 496 (55), 497 (25), 498 (100, $[M+Na]^+$), 499 (18), 500 (75), 501 (12), 502 (10). Theoretically-calculated values were for $C_{17}H_{22}N_3O_3SeBr$: C, 42.96; H, 4.67; N, 8.83. Found: C, 43.22; H, 4.95; N, 9.15.

Assay of superoxide anion-scavenging activities (SOSAs). The superoxide anion-scavenging activities of compounds A**a-d** and B**e-j** were measured by a previously reported method.[23] In brief, hypoxanthine (5 x 10^{-5} M)and ethylendiaminetetraacetic acid (EDTA) (0.1 mM) were prepared in 3 mL of 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer, pH 7.8 **(**the standard solution), with or without various concentrations of compounds A**a-d** and B**e-j**. To evaluate certain superoxide anion-scavenging activities, we prepared a calibration curve using standard solutions of SOD (0.6–30 ng/mL). Compounds A**a-d** and B**e-j** (25 mM) were dissolved in dimethyl sulfoxide and stored at -80^{o}C prior to use. Chemiluminescence was measured with a luminometer (Aloka, BLR201) at 25ºC. The reaction was initiated by the addition of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride (5.8 x 10^{-7} M) to the standard in solution excluding xanthine oxidase, continued for 2.5 minutes, and then for a further 2 minutes after the addition of xanthine oxidase (6.5U). A typical result for compound A**a** is shown in Fig. 1. superoxide anion-scavenging activity was found to be too strong when the compounds were tested primarily at 166 μM; thus the activity was measured at 8.3, 16.6, 33.3 or 41.0 μM. The percent inhibition of 2-methyl-6-(4-methoxyphenyl)-3,7- dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride-dependent chemiluminescence was calculated as previously described [23]. The concentration for 50% inhibition (IC_{50}) was calculated from the values obtained at four different concentrations of compound A**a** (8.3, 16.6, 33.3 and 41.0 μM).

Measurement of uric acid generation in hypoxanthine-xanthine oxidase system. The amounts of uric acid were measured using a uric acid measurement kit (L type WAKO UA M; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Cell culture. In the present study, we used rat pheochromocytoma PC12 cells, because previous reports suggested that this cell line is a useful tool as various models of neurological dysfunctions.[24] PC12 cells were cultured as described previously.[25] In brief,

the cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated horse serum (HS; Gibco BRL, Grand Island, NY) and 5% heat-inactivated fetal bovine serum (FBS, Sanko Junyaku, Co., Ltd., Tokyo, Japan) (serum-containing medium) or in DMEM supplemented with 1% bovine serum albumin (BSA) (serum-free medium).

Detection of phosphorylated proteins. Each compound was suspended in the serum-free medium, and sonicated until fully emulsified. PC12 cells were seeded at 2×10^6 cells/well onto collagen-coated 6-well plates (Corning Incorporated Life Sciences, Lowell, MA) in the serum-containing medium, and precultured for 2 days at 37˚C in an atmosphere of 95% air/5% CO_2. The cells were then washed with phosphate-buffered saline (PBS), and incubated with the abovementioned culture medium containing the various agents shown in Fig. 2 for 10 minutes at 37˚C. The culture plates were then placed on ice and each well was washed with 3 mL of 2mM Tris-HCl buffer (pH 8.0) containing 0.33 M NaF and 6.25 M Na_3VO_4, and subsequently lysed with 150 μL of 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (w/v), 1% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (SDS) (w/v), 50 mM NaF, 0.1% aprotinin (w/v), 0.1% leupeptin (w/v), 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonylfluoride (PMSF). Cell lysates were collected by using a cell scraper, and centrifuged at 15000 x *g* for 30 minutes at 4˚C. The supernatant was collected, and the overall protein concentration was determined by a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with BSA as the standard.

Supernatant fluids containing proteins (20 μg) were mixed with lithium dodecyl sulfate (LDS) sample buffer (Invitrogen Corp, Carlsbad, CA) and incubated for 5 minutes at 80˚C. Proteins in samples were separated on SDS-polyacrylamide gel electrophoresis, and the proteins in gels were electroblotted onto polyvinylidene fluoride (PVDF) filters (Fluorotrans membrane W, 0.2 μm; Nihon Genetics, Tokyo, Japan). Immunoblotting analysis was performed by using monoclonal antibodies against p44/42 ERK or phospho p44/42 ERK (Cell Signaling Technology, Lake Placid, NY) as primary antibodies, followed by reaction with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies from Promega Co.

(Fitchburg, WI, USA) as the secondary antibody. The blots were developed by the enhanced chemiluminescence method (Hyperfilm-ECL plus, Amersham Biosciences Corp., Piscataway, NJ).

The measurement of cytotoxicity by 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-htetrazolium bromide (MTT) assay. PC12 cells were cultured onto collagen-coated 96-well plates (2×10^6 cells/well) in serum-containing medium for 2 days at 37˚C in an atmosphere of 95% air/ 5% CO_2. Culture medium was replaced with 50 μL of the serum-free medium containing each test agent after washing with PBS, and the cells were cultured with each test agent for a range of days. The cytotoxicity was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) reduction assay.[26] The cells were incubated with 0.25 ng of MTT/mL (final concentration) for 2 hours, and the reaction was stopped by adding 50 μL of 50% (v/v) dimethylformamide (DMFA) containing 20% (w/v) SDS. The amount of MTT formazan product was determined photometrically using a micro plate reader (Model Ultrospec Visible Plate Reader II of Amersham Biosciences., Tokyo, Japan) by measuring the absorbance at 562 nm with a reference wavelength of 630 nm. The relationships between absorbance and cell numbers were clarified, and the number of cells in each well was calculated.

Measurement of H_2O_2 concentrations

The concentration of H_2O_2 was determined by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin method with minor modifications[27]. Briefly, compound Aa or ebselen (100 μM) and H_2O_2 (400 μM) were added to 100 μL of distilled water or serum-free medium in a 96-well plate followed by 2h-incubation at 37˚C. Scopoletin (10 μM) and horseradish peroxidase (50μg) were added and. the decrease in scopoletin fluorescence was measured using a microplate fluorometer (1420 Multilabel counter, ARVO; excitation and emission at 335 and 460 nm, respectively; Wallac, Turuk, Finland).

Statistical analysis. The results were expressed as means ±standard deviation (SD). The significance of the differences between the groups compared was determined using the

analysis of variance (ANOVA) followed by Dunnett's test.

RESULTS

Superoxide anion-scavenging activities of compound A, B and ebselen.

The chemical structures, superoxide anion-scavenging activities and IC_{50} values of compounds A**a-d** and B**e-j** and ebselen are shown in Table 1. The superoxide anion-scavenging activities were nearly dose-dependent for all compounds, and A**a**, A**c** and A**d** showed the highest level of superoxide anion-scavenging activity at 166 μM. As compound A**a** had the lowest IC_{50} value at 25.9 μM (Table 1), it was selected and used for further experiments. The superoxide anion-scavenging activities of these compounds did not change by the addition of 50μM of U0126, a selective mitogen-activated protein kinase kinase (MEK) inhibitor (data not shown).

We investigated whether compounds A**a-d** and B**e-j** could eliminate superoxide anions generated by xanthine oxidase. For this purpose, the amount of uric acid, which is the by-product of superoxide anions in the mixture for superoxide anion-scavenging activity assay, was measured with or without compound A**a** (data not shown). The generation of uric acid was not affected by the addition of a relatively high concentration of compound A**a** (41.0 μM), suggesting that this compound did not inhibit xanthine oxidase. In addition, the effects of compound A**a** on cultured PC12 cells were investigated.

Effects of compound Aa, ebselen or NGF on the activation of ERK1/2.

The activation of ERK1/2 is one of the checkpoints to assess activation of the classical Ras/MAPK cascade,[28] which is triggered by an engaged tyrosine kinase receptor or G protein-coupled receptor, and results in proliferation and/or differentiation. We previously confirmed that ebselen is an activator of the Ras/MAPK cascade and an initiator of neuronal differentiation. [25] Therefore, we examined whether compounds A and B could activate ERK1/2, and found that ERK1/2 was activated only by compound A**a** (data not shown). Thereafter we focused on the biological activity of compound A**a**.

The effect of ebselen, compound A**a** or nerve growth factor (NGF) on the

phosphorylation of ERK1/2 was examined (Fig. 2). NGF markedly induced phosphorylation of ERK1/2. Compound A**a** and ebselen similarly activated ERK1/2; however, they had a much weaker effect than NGF. On the other hand, phosphorylation of ERK1/2 was not induced in the presence of 400 μM of H_2O_2 (data not shown).

Effects of compound Aa or ebselen on survival of PC12 cells exposed to H_2O_2.

The oxidative stress-induced cytotoxicity was evaluated by determining the viability of PC12 cells exposed to H_2O_2 (Fig. 3). First, we studied the relationship between cell death and H_2O_2 concentration. After two hours addition of 200, 400, 1000 or 2000μM of H_2O_2, cell viability were 100, 36, 8, or 0%, respectively. At 400 μM of H_2O_2, a significant decrease in cell viability was observed, suggesting that the potent oxidative stress-induced cytotoxicity of H_2O_2.

Pretreatment of PC12 cells with ebselen or compound A**a** attenuated this oxidative stress-induced cytotoxicity. However, NGF did not show this effect. Compound A**a** was more potent than ebselen, and a concentration of even 50 μM was enough to suppress H_2O_2-induced cell death completely. However, such protective effects of both compound A**a** and ebselen disappeared under treatment with U0126.

Effects of cell viability of compound Aa, ebselen or NGF on PC12 cells.

Ebselen prevents ischemia- or lead-induced cell death,[1,2] so we compared the effects of compound A**a**, ebselen and NGF on serum deprivation-induced cytotoxicity (Fig. 4). Cytotoxicity occurred on addition of more than 100 μM of ebselen, when tested over 6 days after exposure. Compound A**a** was nontoxic over 6 days at a concentration of 100 μM, but toxic at 1mM in PC12 cells. These results demonstrated that the toxicity of compound A**a** was weaker than that of ebselen. Contrary to these results, NGF increased cell viability after serum deprivation from culture medium, as described previously (Fig. 4C).[29]

DISCUSSION

In the present study, we have systematically synthesized various organic selenium compounds including selenocarbamates, selenoureas, thioureas, tertiary selenoamides,

2-amino-1,3-selenazoles and bis-(2-amino-5-selenazoyl) ketones, and found that these compounds have superoxide anion-scavenging activities, and their IC_{50} values range approximately from 0.1–100 μM.[15-17,30,31] From the results shown in Table 1, it was confirmed that the superoxide anion-scavenging activities of these newly synthesized compounds, 2-selenoxo-1,3-thiazolidin-4-ones compounds A**a-d** and some of the 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylates compounds B**e-j** were similar to those of other previously synthesized organic selenium compounds such as selenocarbamates and selenoureas. The results suggest that compound Aa and some of the B compounds are organic selenium compounds and useful reactive oxygen species scavengers. The generation of uric acid was not significantly affected by the addition of a relatively high concentration of compound A**a** (41.0 μM), suggesting that our compounds act as scavengers of superoxide anions rather than inhibitors of xanthine oxidase.

A low concentration of O_2^- in the human body generally plays a beneficial role in biological defenses and intercellular signal transduction.[5] On the other hand, excessive O_2^- production is involved in the pathogenesis of a number of disorders, including inflammation, rheumatoid arthritis and asthma.[32,33] Oxidative stress may be defined as an imbalance between the cellular production of reactive oxygen species, a key component of inflammation and inflammatory disorders, and antioxidant defense mechanisms.[5] The processes associated with inflammatory responses are complex and often involve the production of reactive oxygen species including O_2^-. In this study, our newly-synthesized compounds acted as effective O_2^- scavengers *in vitro*. Thus, these compounds may eliminate excessive O_2^- and lead to suppression of reactive oxygen species overproduction.

Compound A**a** and ebselen activated ERK1/2, although their effect was much weaker than that of NGF (Fig. 2). Both compound A**a** and ebselen suppressed H_2O_2-induced cytotoxicity (Fig.3), but this effect was inhibited by pretreatment with U0126. It was confirmed that activation of the MAPK cascade is required for suppression of cytotoxicity by compound A**a** and ebselen. However, H_2O_2-induced cytotoxicity was not inhibited by NGF. As NGF is known to activate the MAPK cascade strongly,[34,35] it was suggested that activation of only the MAPK cascade was

insufficient for suppression of H_2O_2-induced rapid cytotoxicity, and another unknown factor(s) is required.

It is known that serum deprivation induces apoptosis in PC12 cells.[25] Although NGF increased cell viability under serum-free cultivation, compound Aa and ebselen did not suppress serum deprivation-induced cell death. Recent studies suggested that upregulation of Nuclear factor-kappa B [36] or inhibition of p38 mitogen-activated protein kinase (p38 MAPK) and Jun-amino-terminal kinase (JNK) activation [37] suppressed apoptosis induced by H_2O_2 in PC12 cells. Therefore, it might be that both compound A**a** and ebselen can inhibit rapid apoptosis, such as that induced by H_2O_2. On the other hand, although NGF induced phosphorylation of Akt, one of a serine/threonine protein kinase that plays a key role in apoptosis-inhibition, and JNK 2/3, the addition of compound A**a** was irrelevant to the phosphorylation of Akt or JNK 2/3 (data not shown). From this, it was supposed that Aa suppressed cytotoxicity by a different mechanism to that of NGF.

It was suggested that H_2O_2 can induce cell death by apoptosis in PC12 cells.[38] In the present study, vesicles were formed and nucleus fragmentation was observed by addition of Hoechst 33342 solution in the dead cells; therefore, H_2O_2 (400 μM)-induced cell death in the cells may be apoptosis. On the other hand, serum deprivation induced apoptosis in PC12 cells.[25] Because H_2O_2-induced cell death was suppressed by Aa but serum deprivation-induced cell death was not, of the several types of apoptosis, Aa may suppress H_2O_2-induced apoptosis in PC12cells.

We examined whether Aa or ebselen (100 μM) could react with H_2O_2 (400 μM). After a 2-h reaction, the concentration of H_2O_2 did not change, demonstrating Aa or ebselen did not directly react with H_2O_2. Next we examined whether Aa or ebselen could react with H_2O_2 (400 μM) in culture medium with PC12 cells. PC12 cells were treated for 10 minutes with 100 μM of Aa following which the cells were removed and washed with PBS. The cells were then exposed to 400μM of H_2O_2 for 2 hours. The H_2O_2 concentration of the supernatant was similar with or without Aa**.** To exclude the effects of serum in these experiments, a control group utilizing 1% BSA instead of serum was added to the DMEM. H_2O_2 was expected to decrease if Aa or ebselen shows GPX-like activity or if GPX was induced by the addition of Aa or ebselen. Since, the presence or absence Aa or ebselen did not affect the concentration of H_2O_2, Aa or

ebselen was believed to be independent of GPX concentration or GPX-like activity in this study.

Oxidative stress is one of the common causal factors of neuronal cell death.[39] H_2O_2 induced-cytotoxicity was clearly suppressed by compound A**a** and the effect was more potent than that of ebselen. From the results of cytotoxicity measurements (Fig. 4), it was confirmed that the toxicity of compound A**a** was lower than that of ebselen. It appears that the toxicity of compound A**a** will not hinder its usage as a drug, and its clinical application against oxidative stress is hopeful.

Antioxidants such as ebselen regulate intercellular signaling via regulation of kinase and phosphatase.[19] In this study, we found that compound A**a** showed superoxide anion-scavenging activity, induction of ERK1/2 phosphorylation and suppression of H_2O_2-induced cell death. It has been suggested that H_2O_2-induced cell death is partially associated with ERK phosphorylation.[40-42] However, this mechanism may not be exactly known at present. To address these relationships, further studies may be needed.

On the other hand, NGF did not suppress H_2O_2-induced cell death, although ERK1/2 proteins in the PC12 cells were phosphorylated, and serum deprivation-induced apoptosis was suppressed. Thus, it was assumed that compound Aa activated another pathway in addition to the MAPK cascade. Both compound A**a** and NGF inhibited cell death. However, since the mechanisms were different, complementary activity could be expected by using both together. Moreover, A**a** might be able to cross the blood-brain barrier because of its low molecular weight compared with NGF. However, the potential of compound A**a** to prevent and treat some neurological disorders seems to be high enough to warrant future evaluation because the promotion of morphological differentiation was not observed in PC12 cells with the addition of A**a**.

In this study, we found that 3-(2,6-dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one, compound A**a,** had the highest superoxide anion-scavenging activity among the related compounds tested. Furthermore, it was clarified that compound A**a** activated ERK1/2 of cultured PC12 cells and suppressed H_2O_2-induced cytotoxicity. However, the cytotoxicity of compound A**a** was lower than that of ebselen, an analogous active selenium-containing compound. Compound Aa has interesting medical potential to protect against and treat some

neurological disorders, and may even be effective in treating depression induced by stress.

REFERENCES

1. Sarker KP, Biswas KK, Rosales JL et al. Ebselen inhibits no-induced apoptosis of differentiated pc12 cells via inhibition of ask1-p38 mapk-p53 and jnk signaling and activation of p44/42 mapk and bcl-2. *J Neurochem.* 2003;87(6):1345-1353.

2. Hsu PC, Guo YL. Antioxidant nutrients and lead toxicity. *Toxicology.* 2002;180(1):33-44.

3. Kim SM, Kim JI. Decomposition of biological macromolecules by plasma generated with helium and oxygen. *J Microbiol.* 2006;44(4):466-471.

4. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem.* 1995;64:97-112.

5. Kimura H, Sawada T, Oshima S, Kozawa K, Ishioka T, Kato M. Toxicity and roles of reactive oxygen species. *Curr Drug Targets Inflamm Allergy.* 2005;4(4):489-495.

6. Ricci JE, Gottlieb RA, Green DR. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J Cell Biol.* 2003;160(1):65-75.

7. Veloso CA, Isoni CA, Borges EA et al. Inhibition of ros production in peripheral blood mononuclear cells from type 2 diabetic patients by autologous plasma depends on akt/pkb signaling pathway. *Clin Chim Acta.* 2008;394(1-2):77-80.

8. Ruiz-Ramos R, Cebrian ME, Garrido E. Benzoquinone activates the erk/mapk signaling pathway via ros production in hl-60 cells. *Toxicology.* 2005;209(3):279-287.

9. Wendel A, Tiegs G. A novel biologically active seleno-organic compound. Vi. Protection by ebselen (pz 51) against galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol.* 1986;35(13):2115-2118.

10. Jeong DW, Kim TS, Chung YW, Kim IY. Selenoprotein w is a glutathione-dependent antioxidant in vivo. *FEBS Lett.* 2002;517(1/3):225-228.

11. Tiano L, Fedeli D, Santroni AM, Falcioni G. Effect of three diaryl tellurides, and an organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro. *Mutat Res.* 2000;464(2):.269-277.

12. Engman L. Expedient synthesis of ebselen and related compounds. *J Org Chem.* 1989;54(12):2964-2966.

13. Zhang M, Nomura A, Uchida Y et al. Ebselen suppresses late airway responses and airway inflammation in guinea pigs. *Free Radic Biol Med.* 2002;32(5):454-464.

14. Nakamura Y, Feng Q, Kumagai T et al. Ebselen, a glutathione peroxidase mimetic seleno-organic compound, as a multifunctional antioxidant. Implication for inflammation-associated carcinogenesis. *J Biol Chem.* 2002;277(4):2687-2694.

15. Takahashi H, Nishina A, Fukumoto RH, Kimura H, Koketsu M, Ishihara H. Selenocarbamates are effective superoxide anion scavengers in vitro. *Eur J Pharm Sci.* 2005;24(4):291-295.

16. Takahashi H, Nishina A, Fukumoto RH, Kimura H, Koketsu M, Ishihara H. Selenoureas and thioureas are effective superoxide radical scavengers in vitro. *Life Sci.* 2005;76(19):2185-2192.

17. Takahashi H, Nishina A, Kimura H, Motoki K, Koketsu M, Ishihara H. Tertiary selenoamide compounds are useful superoxide radical scavengers in vitro. *Eur J Pharm Sci.* 2004;23(3):207-211.

18. Morey M, Serras F, Baguna J, Hafen E, Corominas M. Modulation of the ras/mapk signalling pathway by the redox function of selenoproteins in drosophila melanogaster. *Dev Biol.* 2001;238(1):145-156.

19. Nishina A, Sekiguchi A, He Y, Koketsu M, Furukawa S. Ebselen, a redox regulator containing a selenium atom, induces neurofilament m expression in cultured rat pheochromocytoma pc12 cells via activation of mitogen-activated protein kinase. *J Neurosci Res.* 2008;86(3):720-725.

20. Tsukagoshi H, Koketsu M, Kato M, Kurabayashi M, Nishina A, Kimura H. Superoxide radical-scavenging effects from polymorphonuclear leukocytes and toxicity in human cell lines of newly synthesized organic selenium compounds. *Febs J.* 2007;274(23):6046-6054.

21. Sommen G, Linden A, Heimgartner H. Synthesis of 2-selenoxo-1,3-thiazolidin-4-ones and 2-selenoxo-1,3-thiazinan-4-ones from isoselenocyanates. *Heterocycles.* 2005;65(8):1903-1915.

22. Favero F, Sommen G, Linden A, Heimgartner H. Synthesis of

5-selenoxo-1,2,4-triazole-1-carboxylates from isoselenocyanates and azodicarboxylates. *Heterocycles.* 2006;67(2):749-762.

23. Kimura H, Nakano M. Highly sensitive and reliable chemiluminescence method for the assay of superoxide dismutase in human erythrocytes. *FEBS Lett.* 1988;239(2):347-350.

24. Shirane M, Nakayama KI. Protrudin induces neurite formation by directional membrane trafficking. *Science.* 2006;314(5800):818-821.

25. Nishina A, Kimura H, Sekiguchi A, Fukumoto RH, Nakajima S, Furukawa S. Lysophosphatidylethanolamine in grifola frondosa as a neurotrophic activator via activation of mapk. *J Lipid Res.* 2006;47(7):1434-1443.

26. Heo HJ, Cho HY, Hong B et al. Protective effect of 4',5-dihydroxy-3',6,7-trimethoxyflavone from artemisia asiatica against abeta-induced oxidative stress in pc12 cells. *Amyloid.* 2001;8(3):194-201.

27. Corbett JT. The scopoletin assay for hydrogen peroxide. A review and a better method. *J Biochem Biophys Methods.* 1989;18(4):297-307.

28. Sturgill TW, Ray LB, Erikson E, Maller JL. Insulin-stimulated map-2 kinase phosphorylates and activates ribosomal protein s6 kinase ii. *Nature.* 1988;334(6184):715-718.

29. Zheng Y, Zhang L, Lu Q et al. Ngf-induced tyro3 and axl function as survival factors for differentiating pc12 cells. *Biochem Biophys Res Commun.* 2008;378(3):371-375.

30. Sekiguchi A, Nishina A, Kimura H et al. Superoxide anion-scavenging effect of 2-amino-1,3-selenazoles. *Chem Pharm Bull (Tokyo).* 2005;53(11):1439-1442.

31. Sekiguchi A, Nishina A, Kimura H et al. Bis-(2-amino-5-selenazoyl) ketone as a superoxide anion-scavenger. *Biol Pharm Bull.* 2006;29(7):1404-1407.

32. Vignola AM, Chanez P, Campbell AM et al. Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med.* 1998;157(2):403-409.

33. Kato M, Hayashi Y, Kimura H. Oxygen radicals in inflammation and allergy related to viral infections. *Curr Drug Targets Inflamm Allergy.* 2005;4(4):497-501.

34. Lillien LE, Claude P. Nerve growth factor is a mitogen for cultured chromaffin cells. *Nature (Lond).* 1985;317(6038):632-634.

35. Go´Mez N, Cohen P. Dissection of the protein kinase cascade by which nerve growth factor activates map kinases. *Nature (Lond).* 1991;353(6340):170-173.

36. Zhang M, Guo RX, Mo LQ et al. Nuclear factor-kappab mediates cytoprotection of hydrogen peroxide preconditioning against apoptosis induced by oxidative stress in pc12 cells. *Clin Exp Pharmacol Physiol.* 2009;36(3):304-311.

37. Cho ES, Lee KW, Lee HJ. Cocoa procyanidins protect pc12 cells from hydrogen-peroxide-induced apoptosis by inhibiting activation of p38 mapk and jnk. *Mutat Res.* 2008;640(1-2):123-130.

38. Jang JH, Surh YJ. Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (pc12) cells. *Mutat Res.* 2001;496(1-2):181-190.

39. Kaur N, Lu B, Monroe RK, Ward SM, Halvorsen SW. Inducers of oxidative stress block ciliary neurotrophic factor activation of jak/stat signaling in neurons. *J Neurochem.* 2005;92(6):1521-1530.

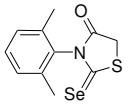
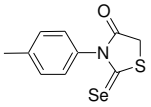
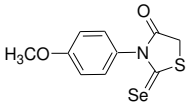
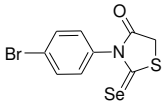
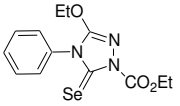
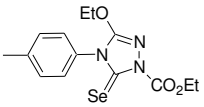
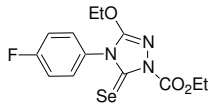
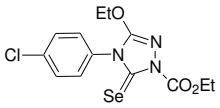
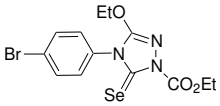
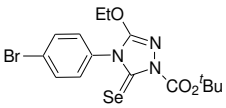
40. Lee DE, Kang NJ, Lee KM et al. Cocoa polyphenols attenuate hydrogen peroxide-induced inhibition of gap-junction intercellular communication by blocking phosphorylation of connexin 43 via the mek/erk signaling pathway. *J Nutr Biochem.*21(8):680-686.

41. Kang KA, Lee KH, Zhang R et al. Protective effects of castanopsis cuspidate through activation of erk and nf-kappab on oxidative cell death induced by hydrogen peroxide. *J Toxicol Environ Health A.* 2007;70(15-16):1319-1328.

42. Zhuang S, Yan Y, Daubert RA, Han J, Schnellmann RG. Erk promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of akt in renal epithelial cells. *Am J Physiol Renal Physiol.* 2007;292(1):F440-447.

Table 1. Chemical structures, superoxide anion-scavenging activities and IC_{50} values of 2-selenoxo-1,3-thiazolidin-4-ones 1, 3-alkoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole- 1-carboxylates and ebselen.

IC_{50} values were determined using linear regression analysis of the dose-response curves of the inhibition of chemiluminescence by the compounds. The superoxide anion-scavenging activities were calculated by a rate of decrease in chemiluminescence value after addition of xanthine oxidase. The superoxide anion-scavenging activities of each compound was expressed as a percentage of that of compound A**a**..

Compound		Inhibition (%) at 166 μM	IC_{50} (μM)
	A**a**	100.0	25.9
	A**b**	44.0	192
	A**c**	100.0	52
	A**d**	100.0	47.3
	B**e**	38.3	134
	B**f**	40.0	287
	B**g**	51.9	160
	B**h**	0.0	-
	B**i**	38.7	299
	B**j**	0.0	-

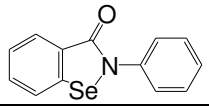
	EB	100.0	20

Figure legends

Fig. 1 Effect of compound A**a** on 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride (MCLA)-dependent luminescence. A, Chemiluminescence inhibition curves by compound A**a**. Arrows indicate the addition of MCLA or xanthine oxidase (XOD). B, Concentration-dependent change in superoxide anion-scavenging activity to determine the IC_{50} value of compound A**a**.

Fig. 2 Effects of compound A**a,** ebselen or NGF on the activation of ERK1/2.

The final concentration of each reagent used for the culture is shown. The phosphorylated ERK1/2 and ERK1/2 proteins were visualized by Western blot, and the intensity of each band was densitometrically calculated. Ratios of the relative intensity of the phosphorylated and non-phosphorylated ERK1 or 2 bands are expressed as the means ± SD of three independent determinations, after normalization at 1 of the values obtained for vehicle-treated control samples. The control group is indicated as o. Levels of significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

Fig. 3 Effects of compound A**a** or ebselen on survival of PC12 cells exposed to H_2O_2.

PC12 cells were treated with compound A**a** for 10 minutes, ebselen or NGF, and then the cells were washed with PBS. The cells were then exposure to 400μM of H_2O_2 for 2 hours. In some experiments, the cells were pretreated for 4 hours with U0126 (50 μM) before treatment with the reagents. The amount of surviving cells was measured by MTT assay as described in the text. Results shown are means ± SD (n=3). The significance of differences between the groups compared was determined using the analysis of variance (ANOVA) followed by Dunnett's test. The control group is indicated as o. Levels of significance: ***, $p<0.005$; -, no significant difference.

Fig. 4 Effects of cell viability of compound A**a**, ebselen or NGF on PC12 cells.

The PC12 cells were cultured for several days in DMEM containing 1% BSA and various concentrations of compound A**a** (A), ebselen (B) or NGF(C). The amount of cells in the well was measured by MTT assay as described in the text. Results shown are means ± SD (n=3).

Fig. 1 Nishina et al.

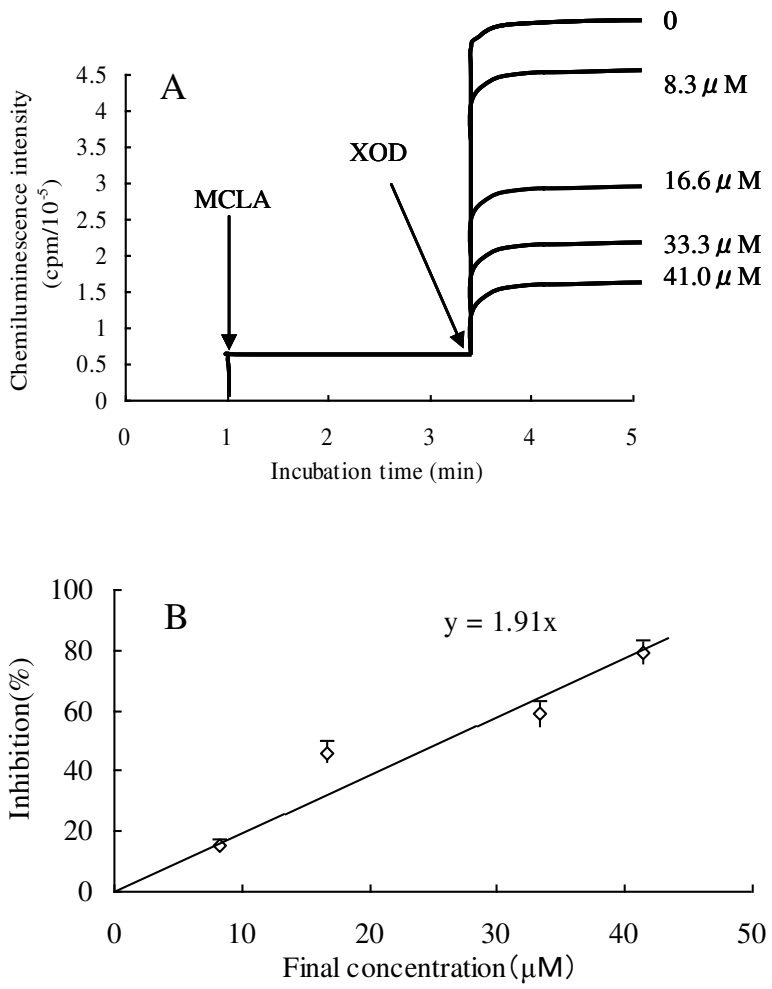
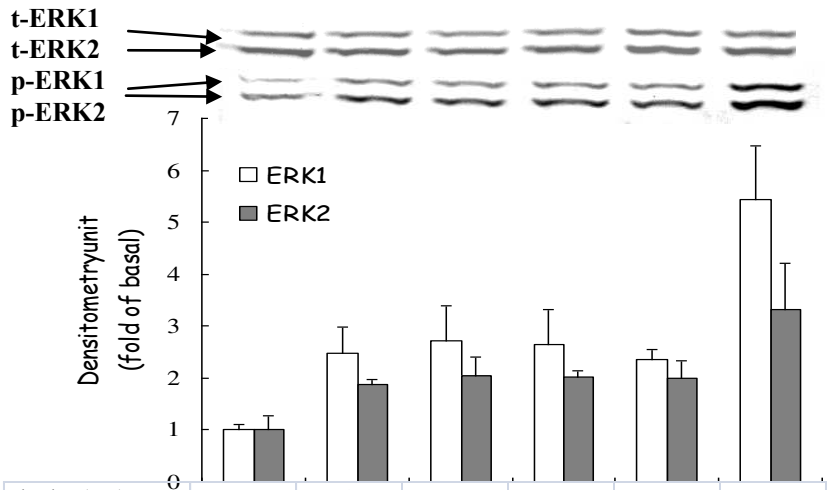
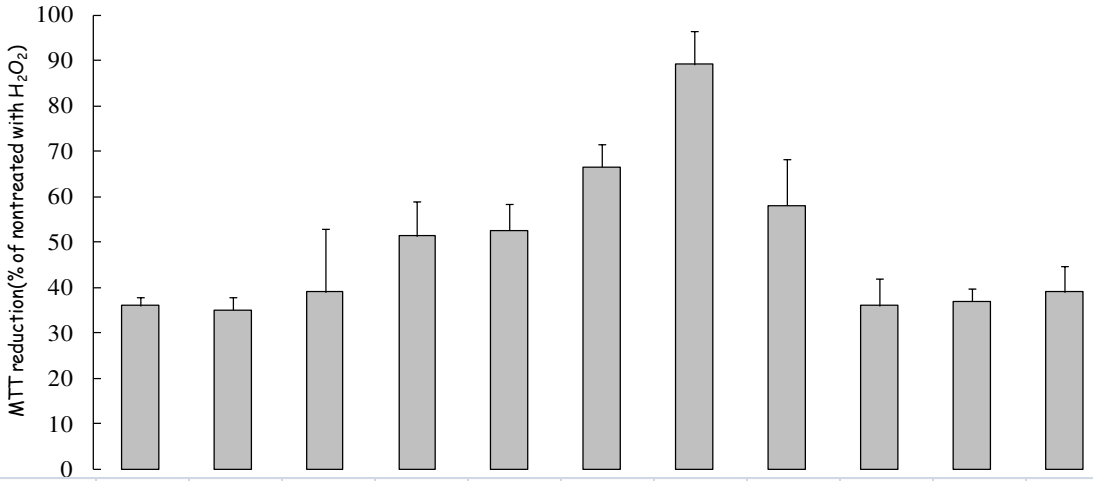


Fig. 2 Nishina et al.



Ebselen (μM)		50	100			
compound A**a** (μM)				50	100	
NGF (ng/ml)						10
ERK1	O	* *	* *	* *	* *	* * *
ERK2	O	*	* * *	*	*	* * *

Fig. 3 Nishina et al.



ebselen (μM)			10	50	100				50		
compound A**a** (μM)						10	50	100		50	
NGF (ng/ml)											10
U0126 (μM)		50							50	50	
	○	–	–	***	***	***	***	***	–	–	–
				○					***		
							○			***	

Fig. 4 Nishina et al.